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ORIGINAL ARTICLE

# First records of *Phytophthora* spp. based on DNA analysis in Lithuania

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## Abstract

The assessment of alien invasive species of *Phytophthora* genus causing serious forest tree species diseases was carried out in Lithuania. The presence of *Phytophthora* DNA was recorded for the first time using real-time PCR analysis on 23 DNA samples. The sampling included wood from diseased trees, leaves from shrubs, leaves baited in water, and soil samples taken around diseased plants. Extracted DNA from soil and plant tissues was tested for the presence of *Phytophthora*. All analysed samples were positively recognized by *Phytophthora*-specific probe during real-time PCR which proved the presence of pathogens in environmental samples.

## Key words

*Oomycetes*, alien invasive species, environmental samples, *Phytophthora* specific DNA probes, baiting, qPCR

## Introduction

*Phytophthora* is plant-damaging genus belonging to *Oomycetes* which are capable to cause enormous economic losses of crops, as well as environmental damage in natural ecosystems. The *Phytophthora* became widely known after 1875, when a new pathogenic agent of potato late blight disease was identified (Bourke 1991). In response to the Irish potato famine the plant pathology was born (Erwin and Ribeiro 2005).

So far, more than 100 *Phytophthora* species have been described and it is likely that worldwide 200–600 species exist being still unknown (Brasier 2009). In 1999, eleven from ca. 55 known species (20%) were considered to be damaging factors to forests and natural ecosystems. However, this proportion changed drastically after the year 2000 and the number of species recognized as potentially damaging tree increased up to 60% (Brasier 2009). It was shown that decline of many forest tree species in Europe and other continents was connected with pathogenic *Phytophthora* acting as inciting factor (Erwin and Ribeiro 2005; Jung et al. 2009). Therefore, there is an urgent need to assess the spread of already known *Phytophthora* spe-

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cies spatially and among different host trees. In Poland as in other European countries there are phenomena of broad lived stands decline driven by root infection caused by pathogenic *Phytophthora* (Jung et al. 1996). In beech decline phenomenon *P. plurivora* and *P. cambivora* are involved. In declining oak stands *P. quercina*, *P. plurivora* and *P. cactorum* were identified as responsible for fine root damage up to 90% (Jung et al. 1999).

So far three species of *Phytophthora* have been identified on rhododendrons in Lithuania: *P. citricola* in 2002, *P. cactorum* in 2004, and *P. ramorum* in 2007 (Jovaišienė 2004; Jovaišienė and Lane 2006). However, the spatial spread of identified *Phytophthora* species was not assessed. The authors are not aware of any investigations concerning *Phytophthora* in natural ecosystems in Lithuania.

The aim of this study was to check the presence of alien invasive species of the genus *Phytophthora* in Lithuania. To identify *Phytophthora* species on symptomatic and declining trees and shrubs in greeneries in cities and forests, molecular DNA markers were used. Moreover, the presence of soil borne *Phytophthora* species in the root rhizosphere of diseased plants was monitored aiming at their isolation and identification.

**Tab. 1.** Characteristics of investigated plants and sampling places for water and soil

| No | City, district, place | Tissue | Plant species | Plant age | Symptoms |
|---|---|---|---|---|---|
| 1 | Kaunas, Amaliai | W | *AP* | Y | Stem spots |
| 2 | Kaunas, KBG | W | *SA* | Y | Stem spots |
| 3 | Kaunas, Vydūno av. | W | *AH* | M | Stem spots |
| 4 | Kaunas, Ąžuolynas | W | *SC* | Y | Stem spots |
| 5 | Jurbarkas district, Raudonės Park | W | *QR* | Y | Stem cancer |
| 6 | Jurbarkas district, River Armena | W | *AP* | P | Stem spots |
| 7 | Jurbarkas district, River Armena | W | *SC* | Y | Stem spots |
| 8 | Kaunas, Radvillėnų pl. | W | *PP* | M | Stem spots |
| 9 | Kaunas, KBG | W | *BP* | P | Stem spots |
| 10 | Kaunas, KBG | W | *AG* | M | Stem spots |
| 11 | Alytus, Lake Dailidė | BL | – | – | – |
| 12 | Šventoji, River Šventoji | BL | – | – | – |
| 13 | Klaipėda district, River Skinija | BL | – | – | – |
| 14 | Jurbarkas district, River Armena | BL | – | – | – |
| 15 | Prienai district, pond | BL | – | – | – |
| 16 | Kaunas, KBG nursery | L | *Rh* | Y | Wilting |
| 17 | Kaunas, KBG nursery | L | *Pi* | Y | Leaf spots |
| 1s | Kaunas, KBG nursery | S | *Rh* | Y | Leaf spots |
| 2s | Kaunas, KBG nursery | S | *Rh* | Y | Leaf spots |
| 3s | Kaunas, KBG nursery | S | *Rh* | Y | Leaf spots |
| 4s | Kaunas, KBG nursery | S | *Rh* | Y | Leaf spots |
| 5s | Kaunas, KBG nursery | S | *Pi* | Y | Leaf spots |
| 6s | Kaunas, KBG | S | *AG* | Y | Leaf spots |

*Abbreviations*: W – wood, L – leaves, BL – baited leaves, S – soil, Y – young, P – premature, M – Mature, *AP – Acer platanoides*, *SA – Salix alba*, *AH – Aesculus hippocastanum*, *SC – Salix caprea*, *QR – Quercus robur*, *PP – Prunus padus*, *BP – Betula pendula*, *AG – Alnus glutinosa*, *Rh – Rhododendron* sp., *Pi – Pieris* sp.

## Material and methods

DNA was extracted from 23 samples: 10 from wood of diseased trees, 5 from baited leaves in water, 2 from symptomatic leaves of *Rhododendron* sp. and *Pieris* sp. and 6 from soil taken around the roots of diseased *Rhododendron* plants (Tab. 1). The majority of samples were taken in Kaunas vicinity (Fig. 1) and others in south-western and western Lithuania.

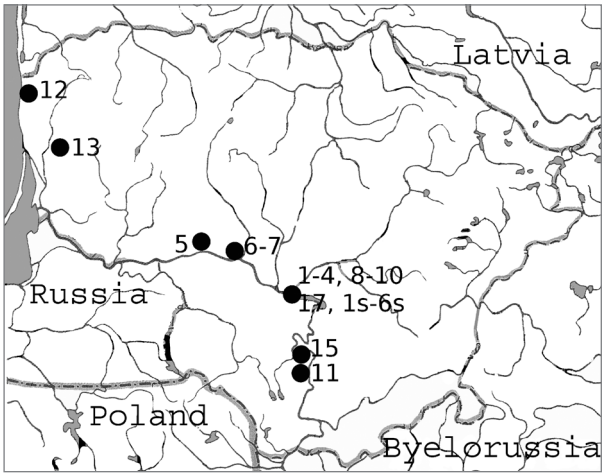


**Fig 1. S**ampling sites in Lithuania scale. The numbers correspond to Table 1, column 1

Investigated trees include *Acer*, *Aesculus*, *Quercus*, *Salix*, *Betula*, and *Alnus* genus visually assessed to be infected by *Phytophthora* spp. (bleeding bark cancer) (Fig. 2–4). Water was taken from rivers and ponds along banks with growing symptomatic trees. Soil was taken from the root rhizosphere of damaged *Rhododendron* sp., *Pieris* sp., and *Alnus glutinosa* growing in Kaunas Botanical Garden (Tab. 1). The symptoms found on *Rhododendron* sp. and *Pieris* sp. included wilting of shoots and spots on leaves. The bleeding bark cancer was the typical symptom of sampled trees.

Soil samples were first checked for the presence of *Pythium* spp. and *Phytophthora* spp. using Alert-LF®, and ELISA tests (Neogen Corporation) followed by baiting (according to Jung et al. 1996) and DNA extraction from infected rhododendron leaves. NucleoSpin® Plant II kit (Mecherey-Nagel) was used to extract DNA from plant tissue (leaves and wood). Due to the fact that wood is difficult to crush, 150 mg of sea sand was added to ensure better homogenization of tissue, as recommended by the manufacturer. The soil samples were pre-baited for four days in a PeaBroth PARP selective media (supplemented with antibiotics) according to the IBL procedure, and the total genomic DNA was extracted using PowerSoil® DNA Isolation Kit (MoBio). The PB-PARP (1000 ml) was prepared by autoclaving 100 g of frozen peas and using the following amendments: 0.25 g of ampicillin, 0.01 g of pimaricin, 0.01 g of rifampicin, 0.05 g of hymexazol, and 0.05 g of PCNB (Erwin and Ribeiro 2005).

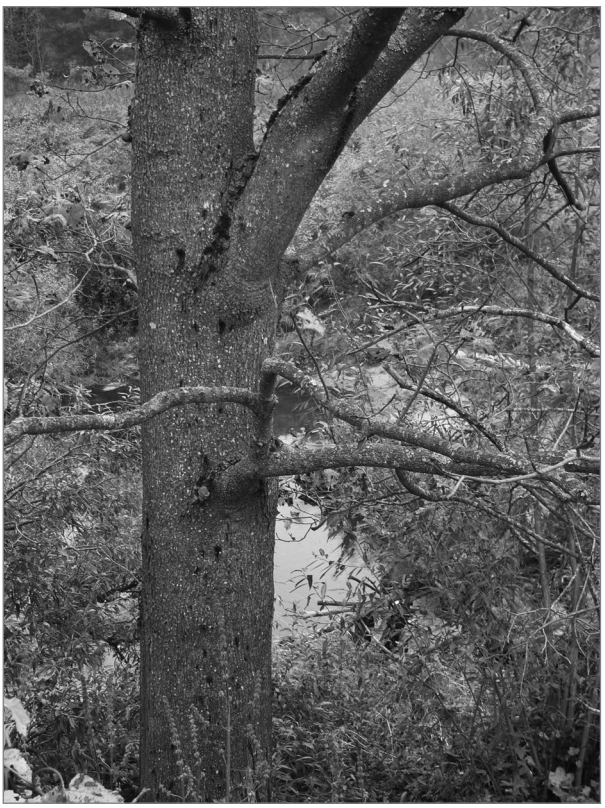

**Fig. 2.** The bleeding spots on bark of *Acer platanoides* in Jurbarkas district (No. Armena2)

Extracted DNA was checked for its quality using spectrophotometer NanoDrop (Thermo Fisher Scientific) and 1% electrophoresis on Tris-EDTA buffer (Sambrook and Russell 2001).

The presence of *Phytophthora* DNA in the samples was confirmed using real-time PCR with a specific TaqMan probe to *Phytophthora* spp. The amplification of the internal transcribed spacer 1 (ITS-1) region was performed with FITS_15Ph, RITS_279Ph, and "All_

**Fig. 3.** The bleeding spots on bark of *Alnus glutinosa* in Kaunas Botanical Garden (No. Kmfj08)

**Fig. 4.** The bleeding cancer on young *Quercus robur* tree in Raudonė Park (No. Raudone4)

**Tab. 2.** Sequences of primers and probe used in the study

| Name | Sequence (5'-3') | Modifications |
|---|---|---|
| FITS_15Ph | TGCGGAAGGATCATTACCACACC | 5' – Phosphorylated |
| RITS_279Ph | GCGAGCCTAGACATCCACTG | 5' – Phosphorylated |
| All_Phytophthora probe | TTGCTATCTAGTTAAAAGCA | 5' – FAM/NFQ-MGB |

*Phytophthora"* probe designed for the genus (Tab. 2) according to Kox et al. (2007). The amplification was carried out in 20 µl reaction using iQ™ Supermix (Bio-Rad Laboratories Inc.) with the primer concentration of 500 nM each and the probe concentration of 83.3 nM.

## Results

The highly variable DNA concentrations of the extracted samples were obtained (Tab. 3), varying from 0.6 to 22.7 ng/ml. The DNA concentrations from the soil samples were more uniform: 5.8–10.0 ng/ml. The ratio of the sample absorbance at 260 and 280 nm varied from 0.76 to 3.26 and the ratio of the sample absorbance at 260 and 230 nm varied from 0.50 to 1.78. The above results confirmed the usefulness of commercial kits for DNA extraction from symptomatic leaves, wood tissue, water and soil.

The ELISA technique performed on our six soil samples as preliminary tests for Oomycetes occurrence turned to be positive for the presence of organisms from both genera of *Pythium* and *Phytophthora* (data not shown). That encouraged us to perform the baiting procedure with rhododendron leaves, and pre-incubation of soil for DNA extraction.

**Tab. 3.** DNA concentrations, quality characteristics of extracted samples, and results of *Phytophthora* specific real time PCR

| No | DNA [ng/ml] | 260/280 | 260/230 | Ct |
|---|---|---|---|---|
| 1 | 9.41 | 1.48 | 0.80 | 26.71 |
| 2 | 11.33 | 1.73 | 1.28 | 27.39 |
| 3 | 3.68 | 2.30 | 0.73 | 26.74 |
| 4 | 1.97 | 3.26 | 0.68 | 26.55 |
| 5 | 22.66 | 1.61 | 1.08 | 26.30 |
| 6 | 3.14 | 1.01 | 0.58 | 28.55 |
| 7 | 4.71 | 1.07 | 0.70 | 25.17 |
| 8 | 3.99 | 1.37 | 0.72 | 29.32 |
| 9 | 0.64 | 0.76 | 0.70 | 24.16 |
| 10 | 4.75 | 1.17 | 0.64 | 19.97 |
| 11 | 2.76 | 2.09 | 1.78 | 21.16 |
| 12 | 5.44 | 1.38 | 0.96 | 27.07 |
| 13 | 6.62 | 1.89 | 0.91 | 26.63 |
| 14 | 1.99 | 2.19 | 0.58 | 26.16 |
| 15 | 9.73 | 1.89 | 1.38 | 16.91 |
| 16 | 2.75 | 2.42 | 0.92 | 17.70 |
| 17 | 5.12 | 1.18 | 0.52 | 24.55 |
| 1s | 6.96 | 1.57 | 0.50 | 25.07 |
| 2s | 10.02 | 1.83 | 0.79 | 25.91 |
| 3s | 7.11 | 1.08 | 0.75 | 20.63 |
| 4s | 5.78 | 2.90 | 1.41 | 25.62 |
| 5s | 6.07 | 2.27 | 0.84 | 10.52 |
| 6s | 7.25 | 2.30 | 0.73 | 20.27 |

All of the 23 investigated samples were recognized by *Phytophthora*-specific probe. Based on this result all of the samples can be considered as containing *Phytophthora* DNA. The amount of DNA in the sample is expressed by the Ct value. The lower the value is, the more *Phytophthora* DNA is present in the sample. Such a situation was observed in the case of three samples: 5s (Ct = 10.52) from leaf spots taken in Kaunas nursery, 15 (Ct = 16.91) from the pond in Prienai district, and 16 (Ct = 17.70) originated from wilting plants in Kaunas nursery (Tab. 1 and 3). In contrast, the lowest amount of patogen DNA was found in water of Šventoji River (sample 12, Ct = 27.07), in maple trees growing along the Armena river (sample 6, Ct = 28.55), and in hackberry wood from Kaunas (sample 8, Ct = 29.32) (Tab. 3).

## Discussion

The currently known distribution in Europe of *Phytophthora* species of threat to forest trees has not been so far reported from Lithuania. Although many species of *Phytophthora* have been identified in European forests to date, including the potentially destructive *P. cinnamomi* and *P. ramorum*, with increased investigation of soil microbial communities in forests, further hitherto unrecognised species have been found. Several species are known to cause severe disease syndromes; other species appear to be involved in pathogen complexes and may be partly responsible for forest decline syndromes (Woodward et al. 2005 and 2010). This study shows presence of *Phytophthora* in the continental climate and considers a potential impact of different *Phytophthora* in the context of forest damage.

The spread of alien pests is of high importance nowadays in Lithuania and the possible pathways between countries and ecosystems should be investigated thoroughly. The presence and spread of pathogens *Phytophthora* genus in natural ecosystems have not been investigated in Lithuania until now. However, the spread of symptomatic trees in parks and riparian ecosystems has been visible in Lithuania already for some time. The spread of *Phytophthora* is accelerated by favourable environmental conditions – soil flooding or excess of moisture, droughts, and temperature extremes (Erwin and Ribeiro 2005). On the other hand, observed more intensive international trade plants for plantings allow the spread of alien species over long distances (Evans and Oszako 2006; Jung et al. 2009).

The visual assessment of disturbed trees with signs of *Phytophthora* infection was carried out in several regions of Lithuania in 2010–2011. The preliminary results indicate that the spread of infection is higher than it was supposed. The most sensitive tree species include the genera of *Acer*, *Alnus*, and *Betula*. The highest number of affected trees was observed in habitats with an excessive soil moisture and along the banks of water

courses (Stančikaitė et al. 2011). The same phenomenon has been observed in Polish riparian ecosystems where decline is caused by *P. alni* subsp. *alni* and *P. alni* subsp. *multiformis* (Oszako 2005).

The extracted DNA concentrations were not high, but in most cases acceptable for further analysis. This could be explained by difficulties to homogenize wood material and small amount of DNA in dead wooden cells. The ratios of the sample absorbance at different wavelengths were variable showing possible presence of contamination (for pure DNA the ratios should be ≥1.8) (NanoDrop user manual 2008).

There is no direct correlation between the amount of DNA in the sample and Ct value (Tab. 3) because the amount of DNA reflects the total DNA from each sample (e.g. plant, microorganisms from soil), while the Ct value reveals the presence of the DNA of the pathogen in question. Hence, it could be assumed that the samples from which the total genomic DNA was extracted contained different amount of *Phytophthora* DNA.

## Conclusions

- The study has shown for the first time the occurrence of pathogens genus *Phytophthora* in 23 samples taken from natural ecosystems and Kaunas Botanical Garden in Lithuania.
- Seventeen samples collected from symptomatic plant tissues revealed the presence of DNA of *Phytophthora* genus, proved by specific probe during real-time PCR analysis.
- The assessment of soil samples taken around diseased plants also indicated the presence of DNA of *Phytophthora* spp.
- DNA of the above pathogens was also present in water samples collected from natural water reservoirs (river, pond and lake).

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